

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



12

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12P 21/02, C12N 15/82 // C07K 14/745		A1	(11) International Publication Number: WO 00/28065
			(43) International Publication Date: 18 May 2000 (18.05.00)
(21) International Application Number: PCT/DK99/00607			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 5 November 1999 (05.11.99)			
(30) Priority Data: PA 1998 01436 6 November 1998 (06.11.98) DK PA 1998 01439 9 November 1998 (09.11.98) DK 60/108,065 12 November 1998 (12.11.98) US			
(71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).			
(72) Inventors; and (75) Inventors/Applicants (for US only): WÖLDIKE, Helle [DK/DK]; Stendyssevej 12, DK-3540 Lyngø (DK). WIBERG, Finn, C. [DK/DK]; Vinkelvej 16, DK-3520 Farum (DK). NIELSEN, Lars, Søgaard [DK/DK]; Nivåpark 58, DK-2990 Nivå (DK).			
(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).			<p>Published</p> <p><i>With international search report.</i></p> <p><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
(54) Title: METHOD FOR THE PRODUCTION OF FVII			
(57) Abstract			
<p>The present invention relates to a method for high efficiency release of recombinant proteins in eukaryotic cells and more specifically, for enhancing the secretion of Factor VII by co-expression of kex2 endoprotease with FVII in cells of mammalian origin.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Method for the production of FVII

Field of the Invention

The present invention relates to a method for high efficiency release of recombinant proteins in eukaryotic cells or more specifically, for enhancing the secretion of Factor VII by co-expression with Kex2 endoprotease in cultured cells of mammalian origin.

Background Of The invention

Advances in cell culture and recombinant DNA technologies have facilitated the expression of a variety of proteins of therapeutic or other economic value using genetically engineered cells. The expression of many biologically active therapeutic proteins, which are derived from higher eukaryotic sources, often requires specific post-translational modifications which do not naturally occur in lower eukaryotic or prokaryotic cells, thus necessitating the use of cells derived from higher eukaryotic sources. For example, the expression of glycoproteins in mammalian cells has the advantage of providing proteins which contain natural glycosylation. Mammalian-produced glycoproteins contain outer chain carbohydrate moieties which are markedly different from the outer chain carbohydrate moieties present on glycoproteins produced from lower eukaryotes. The use of mammalian cells as hosts for the production of secreted mammalian proteins has the significant advantage over secretion from lower eukaryotes in that mammalian cells have a secretory system that readily recognizes and properly processes secretion-directed proteins, which is not necessarily true for lower eukaryotes.

Efficient expression of coding sequences in eukaryotic hosts may also require the expression of associated proteins which are required for the processing, stabilization or modification of the protein to achieve biological activity. Optimal expression of biologically active recombinant proteins may also be dependent upon the presence of specific translation and/or transcription factors. These proteins may be present in host cells at such low levels that efficient expression of recombinant proteins is

limited. Examples of proteins that require specific post-translational modification include certain coagulation factors, which require gamma-carboxylation of specific glutamic acid residues for biological activity and may also require the conversion of specific aspartic acid residues to beta-hydroxy aspartic acid for biological activity.

5

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually give rise to a fibrin clot. Generally, the blood components which participate in the so-called coagulation cascade are proenzymes or zymogens, enzymatically inactive proteins which are converted to proteolytically active enzymes by the action of an activator which in itself is an activated clotting factor. Coagulation factors which have undergone such a conversion and generally referred to as active factors are designated by adding a lower case "a" suffix (e.g. Factor VIIa).

10

Factor VII is a trace plasma glycoprotein that circulates in blood as a single-chain zymogen. The zymogen is catalytically inactive (Williams et al., J. Biol. Chem. **264**, 1989, pp. 7536-7543; Rao et al., Proc. Natl. Acad. Sci. USA **85**, 1988, pp. 6687-6691). Single-chain Factor VII may be converted to two-chain Factor VIIa by Factor Xa, Factor XIIa, Factor IXa or thrombin *in vitro*. Factor Xa is believed to be a major physiological activator of Factor VII. Like several other plasma proteins involved in haemostasis, Factor VII depends on vitamin K for expression in an active form, vitamin K being required for the γ -carboxylation of multiple glutamic acid residues clustered in the N-terminus of the protein. The γ -carboxyglutamic acid (Gla)-containing domain is followed by two domains that are homologous to the epidermal growth factor precursor (EGF) whereas the serine protease part occupies the C-terminal half of the molecule.

20

25

Proteins involved in the coagulation cascade are also often processed to mature proteins by cleavage at dibasic amino acid residues. Thus, Factor VII is synthesized with an N-terminal 38 amino acids propeptide, which is cleaved off C-terminally to two pairs of arginins (R-R-R-R). There are several candidate enzymes which might be involved in this processing *in vivo*, some of which operate preferentially in the en-

30

doplasmic reticulum (ER) and some in the Golgi apparatus in a membrane bound form.

Another important post-translational modification of Factor VII is gamma-carboxylation of 10 glutamic acid residues located close to the cleavage point of the propeptide. The sequence of events is indicated by the fact, that the presence of the propeptide and the correct sequence of it seems important for the process of gamma-carboxylation (Busby et al., Curr. Adv. in Vit. K. Res. 173-181 (1987) and Ulrich et al., J. Biol. Chem. 263 (20) 9697-9702 (1988)), which occurs in the ER catalyzed by a membrane-bound carboxylase.

Kex2 endoprotease of *Saccharomyces* yeast is a protéase which specifically processes the precursor of mating type α -factor and a killer factor. The properties of the Kex2 endoprotease are reported to be the following: (1) Kex2 cleaves at the C-terminal of Lys-Arg sequence for excision of mating type α -factor from its precursor, and at the C-terminal of Lys-Arg sequence and Pro-Arg sequence to release mature killer factor; (2) a purification thereof was attempted, and it was found that the enzyme is present in a membrane fraction and requires calcium ions for activation thereof; (3) Kex2 is a glycoprotein having a molecular weight of 100 to 120 K Dalton; (4) Kex2 specifically cleaves at the C-terminal of sequences Arg-Arg, Lys-Arg, and Pro-Arg (BBRC, 144, 807-814, 1987).

In WO 90/01550 plasmids are disclosed carrying polycistronic expression units including an intercistronic leader. In US 5460950 host cells are disclosed expressing PACE, a human endoprotease, capable of cleaving precursor polypeptides where the PACE encoding sequence and the precursor polypeptide encoding sequence is operably linked to an expression control sequence permitting co-expression of the two sequences.

Summary of the invention

The present invention is related to a method for producing Factor VII comprising

- a) cultivating a mammalian cell line comprising a DNA sequence encoding a yeast endoprotease or a derivative thereof and a DNA sequence encoding FVII in a suitable culture medium and
- b) isolating Factor VII from the medium.

According to one embodiment of the present invention the yeast endoprotease is preferably derived from a *Saccharomyces* strain and is preferably a Kex2 like endoprotease. The yeast endoprotease may be truncated at its C-terminal end thereby being deprived of its transmembrane region. Further an ER retention signal may be added to the C-terminal end of the truncated endoprotease. The yeast endoprotease is preferably the Kex2 endoprotease from *Saccharomyces cerevisiae*.

- The culture medium is preferably a serum free medium and the mammalian cell line is preferably a CHO cell line or a BHK cell line.

Detailed Description of the Invention

Definitions:

- As used herein the term "KEX2" means the yeast endoprotease gene from *S. cerevisiae*.

As used herein the term "Kex2" means the yeast endoprotease gene product from *S. cerevisiae*.

- As used herein the term "full length Kex2" means the complete sequence of Kex2 from amino acid 1 to 814.

- As used herein the term "truncated Kex2" means a Kex2 enzyme where the C-terminal end has been removed, in particular from amino acid 814 to amino acid 614 or 675, respectively.

As used herein the term "ER" means: Endoplasmatic Reticullum.

As used herein the term "ER retention signal" means KDEL at the C-terminal.

5

As used herein the term "a Kex2 like endoprotease" means a yeast endoprotease with an amino acid sequence which has a degree of homology to the amino acid sequence of full length Kex2 of at least about 50%, preferably at least about 80%, more preferably at least about 90%, even more preferably at least about 95%.

10 The amino acid sequences of the Kex2 like yeast endoprotease may differ from the amino acid sequence of full length Kex2 by insertion or deletion of one or more amino acid residues and/or substitution of one or more amino acid residues in the natural sequence by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not
15 significantly affect the folding and/or the activity of the endoprotease.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine and histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine and valine), aromatic amino acids
20 (such as phenylalanine, tryptophan and tyrosine) and small amino acids (such as glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, e.g., by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser,
25 Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

With the term "a derivative of an yeast endoprotease" is meant a C-terminally truncated sequence which may contain an ER retention signal attached to the C-
30 terminal end.

The method according to the present invention generally comprises:

- (a) introducing into the mammalian cell line a vector system(s) comprising DNA encoding an endoprotease and DNA encoding Factor VII ; and
- 5 (b) growing the transfected mammalian cells in an appropriate medium; and
- (c) isolation of Factor VII.

The vector system will preferably comprise two separate vectors being capable of expressing FVII and the endoprotease, respectively. In this embodiment of the present invention, the mammalian cells are co-transfected with the two vectors and then
10 cultured in a suitable culture medium. Alternatively an already established Factor VII expression clone may be transfected with a vector capable of expressing the endoprotease. The vector system may also comprise one single vector comprising the FVII expression cassette and the endoprotease expression cassette.

15

The mammalian cells used as host cells in the method of the present invention include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, HEK 293 cells or any number of other immortalized cell lines available, e.g., from the American Type Culture Collection.

20

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10), CHL (ATCC CCL39), HEK 293 (ATCC CRL 1573) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. **159** (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. **1** (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA **79** (1982), 422 - 426; Wigler et al., Cell **14** (1978), 725; Corsaro and Pearson, Somatic Cell Genetics **Z** (1981), 603, Graham and van der Eb, Virology **52** (1973), 456; and Neumann et al., EMBO J. **1** (1982), 841 - 845.

25

The vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating
5 vector, i.e. a vector which exists as an extra chromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. The vector is preferably an expression vector in which the encoding DNA
10 sequence is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding
15 for the polypeptide.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the
20 transcription of the encoding DNA in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. 1 (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222 (1983), 809 - 814) or the adenovirus 2 major late promoter.

25 The encoding DNA sequence may also, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or the ADH3 (McKnight et al., op. cit.) terminator.

The vector may further comprise elements such as polyadenylation signals (e.g. from
30 SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the

SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

5 The vector will also preferably contain a signal peptide coding region, which codes for an amino acid sequence linked to the amino terminus of the FVII polypeptide which can direct the expressed FVII polypeptide into the cell's secretory pathway of the host cell. The signal may be homologous or heterologous to the host mammalian cell line and it may be the natural signal peptide

10 Finally, the vector may comprise a DNA sequence enabling it to replicate in the host cell in question. An example of such a sequence in a mammalian cell is the SV40 origin of replication.

The transfected mammalian cells are cultured in a suitable nutrient medium under conditions permitting the co-expression of FVII and the endoprotease whereupon
15 FVII is recovered from the culture medium. The medium used to culture the mammalian cells may be any conventional medium suitable for growing mammalian cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). FVII
20 produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography,
25 gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE 1**Construction of KEX2 expression plasmid.**

Fig.1 outlines the construction of the Kex2 expression plasmid using pcDNA3 from Invitrogen as expression cassette.

- 5 From a genomic clone of KEX2 in plasmid pME568, the N-terminal 800 bp's of the coding region was subcloned into pUC19 Acc651-Xba1 after introduction of an Acc651 site 15 bp's upstream of the initial ATG by PCR of the N-terminal 375 bp's using the primers :

10 Downstream : 5' ACCTGGTACCCATTATAAGATGAAAG 3' (Seq ID:1)
Acc651

Upstream : 5' GGTAACAAGCTTIGAGTCC 3' (seq ID: 2)
Hind3

15

- This 375 bp's Acc651-Hind3 fragment was ligated to the adjacent 420 bp's Hind3-Xba1 fragment and cloned into pUC19. From the resulting plasmid, pHW1252, the 800 bp Acc651-Xba1 fragment was excised and inserted with the C-terminal 1750 bp Xba1-SnaB1 fragment from pME568 into pcDNA3 Acc651-EcoRV 5.4 kb to yield the expression plasmid pHW1253. The sequence of KEX2 in pHW1253 is essentially as described in Philippsen, P. et al. Nature 387 (suppl.), 93-98 (1997) in The Yeast Genome Directory.

25 **EXAMPLE 2**

Co-expression of Kex2 with FVII

- Baby Hamster Kidney (BHK) cells expressing FVII (Berkner et al., Cold Spring Harbor Symp. Quant. Biol. 51, 531-541 (1986)) were transfected with 2µg plasmid pHW1253 as described in Example 1, using LipoFect Amine method as described by the supplier (Gibco, Life Technologies, Roskilde, Denmark). Stable clones were se-
- 30

lected in medium (Du Ibecco's modified Eagle's medium, 10 % fetal calf serum , 100 IU penicillin, 100 IU streptomycin , 1mmol/l Na-pyrovate and 5mg/l vitamin K1) containing 1mg/l Geneticin G418 (Gibco) . After selection, stable clones were picked using cloning cylinders and maintained at 37°C in an atmosphere containing 5%

5 CO₂.

Stable clones were screened in a FVII-ELISA (Novo Nordisk) for FVII production.

The Kex2 enzyme activity from 1 million cells of the above clones was determined essentially as described by N.C. Rockwell, G.T. Wang ,G.A. Kraft , and R.S. Fuller. , Biochemistry 36 (7) :1912 - 1917, 1997. The original FVII producing cell line was

10 cultivated in parallel and used as a reference.

We are seeing a positive correlation between Kex 2 expression and secretion of FVII in this BHK cell line, with 2-3 times more FVII produced in the clones co-expressing Kex2.

15 **EXAMPLE 3**

Construction of FVII expression plasmid

A FVII cDNA with deleted non-translated regions was prepared by PCR with Taq polymerase using the following primers:

20 AAC GGA TCC ACC ATG GTC TCC CAG GCC CTC AGG (Seq ID 3)

ACG GAA TTC ACT AGT CTA GGG AAA TGG GGC TCG CAG GA (Seq ID 4)

and the human FVII cDNA as a template (Hagen et al. Proc. Natl. Acad. Sci. USA 83, 1986, pp. 2412-2416). The PCR fragment was cloned into the vector pBluescript

25 II KS+ (Stratagene) and the sequence was verified. The cDNA was transferred as a BamHI-SpeI fragment to the mammalian cell expression vector Zem219b (Busby et al. J. Biol. Chem. 266, 1991, pp. 15286-15292), which carries a mouse metallothionin in promoter for driving the inserted cDNA and the dihydrofolate reductase cDNA driven by an SV40 promoter for use as a selectable marker. The resulting

30 plasmid was designated pLN174.

EXAMPLE 4**FVII-transfection of CHO cells with and without the KEX2 cDNA and measurement of FVII production from transfected cells**

5 CHO-K1 cells (ATCC CCL 61) adapted for growth in suspension were transfected with:

a. The FVII expression plasmid pLN174 (example a) and the expression vector pcDNA3 (Invitrogen) without inserted cDNA.

10

b. The FVII expression plasmid pLN174 (example a) and the pcDNA3/KEX2 expression vector

using the Qiafect transfection reagent. Double transfectants were selected using 1
15 μ M methotrexate and 700 μ g/ml Geneticin (GIBCO). When clones were visible by the naked eye the cultures were transferred to T-flasks for further culture.

Pools of transfectants (a and b as described above) were seeded in T25 flasks (0.25
x 10⁶ cells per flask) for measurement of FVII production. When the cells adhered to
20 the substrate, fresh medium was added containing 5 μ g/ml of vitamin K1. Culture medium was harvested after 2 further days and assayed for FVII activity with an ELISA kit (DAKO). As can be seen from the table below cotransfection of pcDNA3/KEX2 and FVII gives a 5 times increased expression level of FVII immunoreactivity as compared to when FVII is cotransfected with the pcDNA3 vector
25 alone.

Cell type	Plasmids used for transfection	FVII concentration $\mu\text{g/ml}$
a	pLN174/pcDNA3	0.18
b	pLN174/pcDNA3+KEX2	0.90

CLAIMS

1. A method for producing Factor VII comprising a) cultivation of a mammalian cell line comprising a DNA sequence encoding a yeast endoprotease or a derivative thereof and a DNA sequence encoding FVII in a suitable culture medium and b) isolation of Factor VII from the medium.
2. A method according to claim 1, wherein said yeast endoprotease is derived from a *Saccharomyces* strain.
3. A method according to claim 2, wherein said yeast endoprotease is a Kex2 like endoprotease.
4. A method according to claim 3 wherein said yeast Kex2 like endoprotease is C-terminally truncated and has no transmembrane region.
5. A method according to claim 4, wherein said yeast Kex2 like endoprotease has ER retention signal added to the C-terminal end.
6. A method according to claim 2 wherein the yeast endoprotease is the Kex2 endoprotease from *Saccharomyces cerevisiae*.
7. A method according to claim 1, wherein the culture medium is a serum free medium.
8. A method according to claim 1 wherein the mammalian cell line is a CHO cell line.
9. A method according to claim 1 wherein the mammalian cell line is a BHK cell line.

10. A method according to claim 1 wherein the mammalian cell line is a HEK 293 cell line.

1/1

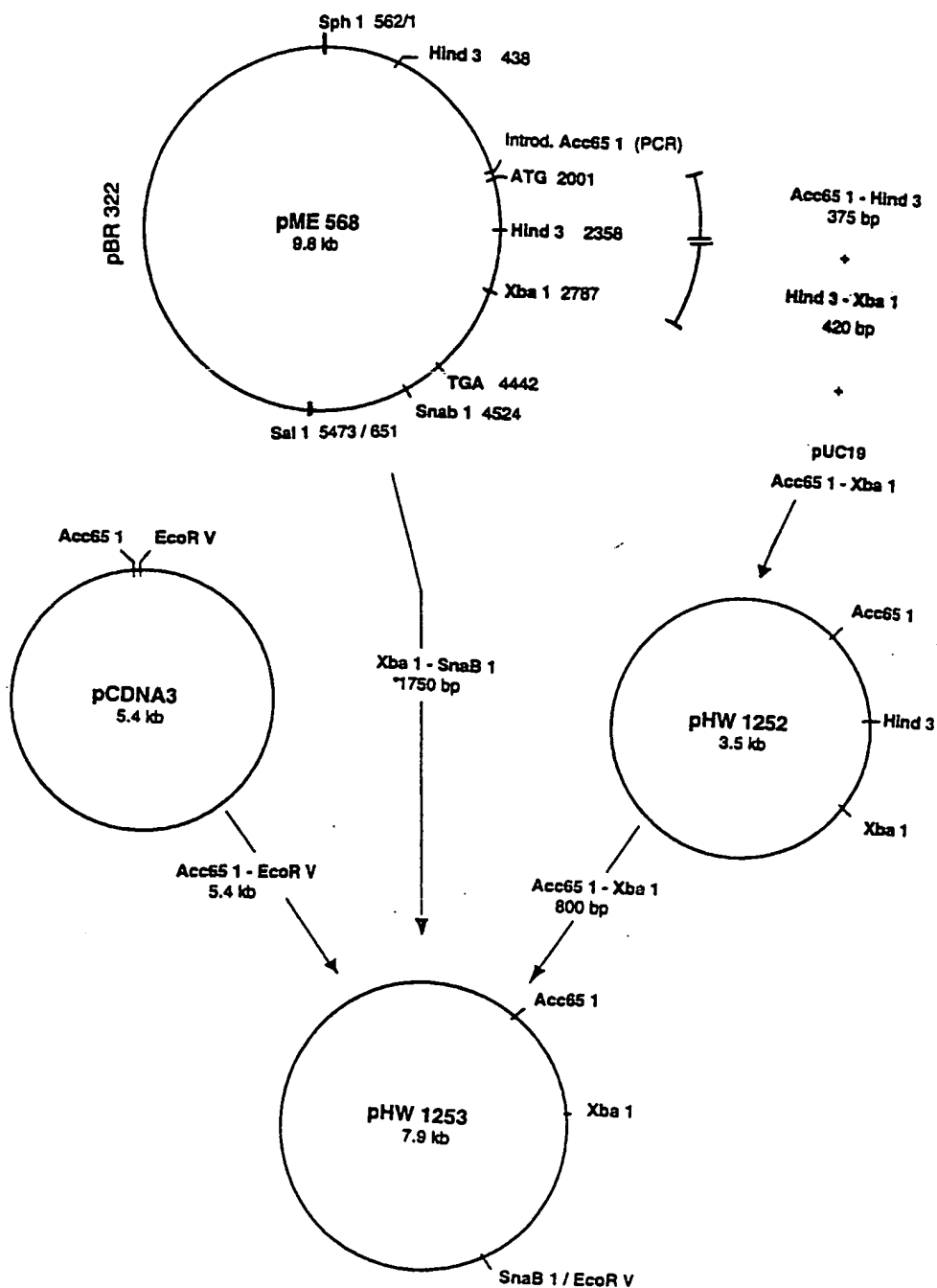


Fig. 1

SEQUENCE LISTING

<110> Novo Nordisk A/S

5 <120> Method for making FVII

<130> 5565seq

<140> 5565

10 <141> 1998-11-06

<160> 4

<170> PatentIn Ver. 2.1

15

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

20

<220>

<223> Description of Artificial Sequence:primer 1

<400> 1

25 acctggtacc ccattataag atgaaag

27

<210> 2

<211> 18

30 <212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer 2

35

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00607

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12P 21/02, C12N 15/82 // C07K 14/745

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12P, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5516650 A (DONALD C. FOSTER ET AL), 14 May 1996 (14.05.96), See column 3, lines 6-29 --	1-10
X	US 5460950 A (PHILIP J. BARR ET AL), 24 October 1995 (24.10.95), See column 5, lines 21-40; column 6, line 12 - column 7, line 23; column 7, lines 48-59; claims 1-4 --	1-10
A	EP 0319944 A2 (ZYMOGENETICS INC.), 14 June 1989 (14.06.89) -----	1-10

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

& document member of the same patent family

Date of the actual completion of the international search

23 February 2000

Date of mailing of the international search report

14-03-2000

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Hampus Rystedt/Els

Telephone No. +46 8 782 25 00

<400> 2

ggtaacaagc ttgagtcc

18

5 <210> 3

<211> 33

<212> DNA

<213> Artificial Sequence

10 <220>

<223> Description of Artificial Sequence:Primer 3

<400> 3

aacggatcca ccatggtctc ccaggccctc agg

33

15

<210> 4

<211> 38

<212> DNA

20 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer 2

25 <400> 4

acggaattca ctagtctagg gaaatggggc tcgcagga

38

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.
PCT/DK 99/00607

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5460950 A	24/10/95	US 5965425 A	12/10/99
		AT 158816 T	15/10/97
		CA 2096418 A	27/05/92
		DE 69127829 D,T	19/03/98
		DK 574402 T	18/05/98
		EP 0574402 A,B	22/12/93
		SE 0574402 T3	
		EP 0785273 A	23/07/97
		ES 2109336 T	16/01/98
		JP 6504435 T	26/05/94
EP 0319944 A2	14/06/89	WO 9209698 A	11/06/92
		SE 0319944 T3	
		AT 111956 T	15/10/94
		AU 651680 B	28/07/94
		AU 1492692 A	29/10/92
		AU 2670688 A	08/06/89
		DE 3851613 D,T	19/01/95
		DK 684388 A	11/08/89
		ES 2059478 T	16/11/94
		FI 885679 A	09/06/89
		JP 2002338 A	08/01/90
		JP 2769170 B	25/06/98
		US 5516650 A	14/05/96
		US 5648254 A	15/07/97

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.

PCT/DK 99/00607

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5516650 A	14/05/96	AT 111956 T	15/10/94
		AU 651680 B	28/07/94
		AU 1492692 A	29/10/92
		AU 2670688 A	08/06/89
		DE 3851613 D,T	19/01/95
		DK 684388 A	11/08/89
		EP 0319944 A,B	14/06/89
		SE 0319944 T3	
		ES 2059478 T	16/11/94
		FI 885679 A	09/06/89
		JP 2002338 A	08/01/90
		JP 2769170 B	25/06/98
		AT 90966 T	15/07/93
		CA 1340263 A	15/12/98
		DE 3786321 A,T	29/07/93
		DK 567087 A	30/04/88
		EP 0266190 A,B	04/05/88
		JP 1085084 A	30/03/89
		JP 2561677 B	11/12/96
		JP 2922461 B	26/07/99
		JP 8252094 A	01/10/96
		KR 9702166 B	24/02/97
		NO 173741 C	26/01/94
		NO 874495 D	00/00/00
		US 4959318 A	25/09/90
		AT 93272 T	15/09/93
		DE 3688900 A,T	23/09/93
		DK 308586 A	28/12/86
		EP 0215548 A,B	25/03/87
		JP 2614848 B	28/05/97
		JP 2688407 B	10/12/97
		JP 9107976 A	28/04/97
		JP 62111690 A	22/05/87
		NO 305660 B	05/07/99
		NO 862601 D	00/00/00